Published in final edited form as:

Vaccine. 2009 April 21; 27(18): 2453–2468. doi:10.1016/j.vaccine.2009.02.048.

Viral load and clinical disease enhancement associated with a lentivirus cytotoxic T lymphocyte vaccine regimen

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Abstract

Effective DNA-based vaccines against lentiviruses will likely induce CTL against conserved viral proteins. Equine infectious anemia virus (EIAV) infects horses worldwide, and serves as a useful model for lentiviral immune control. Although attenuated live EIAV vaccines have induced protective immune responses, DNA-based vaccines have not. In particular, DNA-based vaccines have had limited success in inducing CTL responses against intracellular pathogens in the horse. We hypothesized that priming with a codon-optimized plasmid encoding EIAV Gag p15/p26 with co-administration of a plasmid encoding an equine IL-2/IgG fusion protein as a molecular adjuvant, followed by boosting with a vaccinia vector expressing Gag p15/p26, would induce protective Gag-specific CTL responses. Although the regimen induced Gag-specific CTL in four of seven vaccinated horses, CTL were not detected until after the vaccinia boost, and protective effects were not observed in EIAV challenged vaccinates. Unexpectedly, vaccinates had significantly higher viral loads and more severe clinical disease, associated with the presence of vaccine-induced CTL. It was concluded that 1.) further optimization of the timing and route of DNA immunization was needed for efficient CTL priming in vivo, 2.) co-administration of the IL-2/IgG plasmid did not enhance CTL priming by the Gag p15/p26 plasmid, 3.) vaccinia vectors are useful for lentivirus-specific CTL induction in the horse, 4.) Gag-specific CTL alone are either insufficient or a more robust Gag-specific CTL response is needed to limit EIAV viremia and clinical disease, and 5.) CTL-inducing vaccines lacking envelope immunogens can result in lentiviral disease enhancement. Although the mechanisms for enhancement associated with this vaccine regimen remain to be elucidated, these results have important implications for development of lentivirus T cell vaccines.

Introduction

It is widely accepted that a protective lentivirus vaccine will need to induce broadly neutralizing antibodies in addition to cytotoxic T lymphocytes (CTL) directed against multiple conserved epitopes. Pre-challenge infusions of HIV-1-specific broadly neutralizing antibodies are capable of blocking SHIV infection in rhesus macaques [1–4], suggesting that a vaccine eliciting the

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appropriate broadly neutralizing antibodies in humans could provide complete protection against HIV-1. However, it is also possible that a CTL-inducing vaccine could provide significant lentivirus control in the absence of neutralizing antibodies. The appearance of virus-specific CTL in the peripheral blood is temporally associated with the decline of primary viremia in acutely HIV-1-infected patients, and occurs well before serum neutralizing antibody activity is detected [5,6]. High levels of HIV-1-specific CTL are detected in HIV-1-infected clinical long-term nonprogressors [7], and CTL activity is inversely correlated with viral load [8]. Moreover, loss of HIV-1-specific CTL activity is associated with rapid clinical progression to AIDS [9]. As in HIV-1-infected humans, emergence of CTL in macaques coincides with virus clearance during primary SIV infection [10]. Importantly, depletion of CD8⁺ cells in infected macaques is associated with a rapid increase in viremia [11,12].

Despite the ability of CTL to control lentivirus replication, vaccine-elicited CTL responses have not been protective against HIV-1 in humans. The STEP human phase 2b efficacy trial evaluating Merck's adenovirus serotype 5 (Ad5) vector vaccine that elicited Gag, Pol, and Nef-specific T cell responses, was terminated in September 2007 because it did not prevent infection, apparently led to increased HIV-1 acquisition, and failed to reduce viral load in vaccinees that became infected [13–17]. The STEP trial used an Ad5 prime-Ad5 boost regimen, which was protective against SHIV89.6P challenge in macaque studies [18]. However, this regimen was not protective when macaques were challenged with virulent SIVmac239 [19,20]. Although a DNA prime-Ad5 boost regimen was partially protective against SIVmac239 challenge, protective effects were only observed in macaques sharing the *Mamu-A*01* MHC class I allele [19,20]. These studies not only highlight the significant effects of MHC class I haplotype on T cell vaccine efficacy, but also the critical importance of selecting appropriate nonhuman challenge models for translational lentivirus T cell vaccine research.

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus that causes persistent infection of horses worldwide [21–23]. Recurrent episodes of cell-free viremia, concurrent with fever, lethargy, thrombocytopenia, and anemia (and in some cases, weight loss, ventral edema, petechiation, hemorrhage, and death), generally occur during the first year of EIAV infection. Clinical episodes usually become less severe, and most horses eventually control the infection, remaining inapparent carriers [22,24]. Because adaptive immune responses control infection in most horses [25–27], the EIAV system provides a powerful large animal model in which to dissect basic correlates of protective lentiviral immunity.

As in HIV-1 and SIV, virus-specific CTL are critically important in EIAV control. The initial plasma viremia in acute EIAV infection is terminated prior to the appearance of neutralizing antibody, but concurrent with the appearance of CTL [28–30]. We have used equine MHC class I tetramers to show that EIAV Env- and Gag-specific CD8⁺ cells can be detected 14 days post-infection and can comprise up to 6.7% of nonstimulated circulating CD8⁺ cells [31]. This work has also shown an inverse correlation between Gag-specific CD8⁺ cell frequency and viral load associated with control of EIAV viremia and clinical disease [31]. CTL epitopes have been identified in Gag, Pol, Env, Rev, and in the protein encoded by the S2 open reading frame [32–36]. Importantly, the EIAV Gag p15 matrix and Gag p26 capsid proteins are the most frequently recognized by CTL from inapparent carrier horses [33].

MHC class I diversity is a major obstacle to developing vaccines that will induce CTL responses in a population. One solution to this problem is to target viral proteins that contain overlapping clusters of CTL epitopes that can be presented by several different MHC class I molecules. Overlapping clusters of epitopes recognized by CTL from HIV-1-infected people with different MHC backgrounds have been described [37–40]. We have identified regions in EIAV Gag that contain clusters of epitopes recognized by CTL from several EIAV-infected inapparent carrier horses with diverse MHC class I alleles [36]. Specifically, four epitope clusters occur in Gag

p15 and p26 proteins [36]. In addition, these epitope clusters contain conserved epitopes recognized by high avidity CTL [41]. Since the Gag p15 and p26 proteins are most frequently recognized by CTL from horses that have controlled EIAV infection, and since these same proteins contain epitope clusters recognized by high avidity CTL from MHC class I disparate horses, Gag p15 and p26 are considered important targets for a protective EIAV CTL vaccine.

The best protection against heterologous lentivirus challenge in nonhuman primates has been achieved with attenuated live SIV [42–44]. Live attenuated viruses have also been protective in the EIAV system [45–47]. Despite the high levels of protection achieved by live attenuated lentiviruses, safety considerations rule out an attenuated virus approach to an HIV-1 vaccine since attenuated live SIV eventually causes disease in macaques [48]. Therefore, HIV-1 vaccine development efforts continue to focus on DNA vaccines and replication defective virus vectors. Although DNA vaccines induce strong cell-mediated immunity in mice, DNA vaccines have less immune potency in larger animal models and humans [49]. One approach to augment HIV-1 and SIV-specific immune responses (including CTL) elicited by DNA vaccination is by administration of a plasmid encoding an IL-2/IgG fusion protein [50]. The adjuvant properties of the IL-2/IgG fusion protein result from the functional activity and increased avidity of IL-2, and the long in vivo half life afforded by the IgG heavy chain [50, 51]. The sustained augmentation of DNA vaccine-elicited cellular immune responses provided by the IL-2/IgG plasmid is likely due to enhanced initial priming of T lymphocytes [52]. This strategy has elicited robust *Mamu-A*01*-restricted Gag-specific CTL responses in vaccinated macaques, and has been protective against SHIV89.6P challenge [53]. As stated above however, results of vaccine studies using *Mamu-A*01* macaques and SHIV89.6P challenge have not translated to those obtained following SIV challenge nor observed in human trials [17,19,20].

Neither DNA nor viral vector vaccines that elicit CTL and protect horses against EIAV challenge have been developed. In fact, despite considerable effort there are very few reports of CTL induction in horses against any intracellular pathogen using DNA or viral vector vaccine approaches [54,55]. In addition to providing a unique large animal outbred model in which to dissect the mechanisms of lentivirus immune control, the difficulties in eliciting CTL in horses using vaccine strategies other than live attenuated viruses make them a rigorous translational model species in which to evaluate alternative approaches for induction of lentivirus-specific CTL. To that end, we recently described a DNA vaccination strategy using a plasmid containing codon-optimized EIAV *gag* [54]. This construct elicited EIAV Gag-specific CTL and CD8⁺ T cells as measured by functional CTL assays and tetramer staining [54]. In an effort to augment these CTL responses, a plasmid encoding recombinant equine IL-12 was co-administered with the codon-optimized EIAV *gag* plasmid, but no augmentation was observed [54]. Although CTL responses were induced in this study, the vaccine regimen involved intratracheal injection of the plasmids, a route of administration that would likely be considered impractical for a vaccine. Moreover, CTL responses could not be detected without in vitro stimulation, indicating the frequency of elicited CTL was low. In an effort to improve upon these results, the current study was conducted to test the hypothesis that intramuscular/intradermal priming with a codon-optimized plasmid encoding EIAV Gag p15/p26 with co-administration of a plasmid encoding an equine IL-2/IgG fusion protein as a molecular adjuvant, followed by intradermal boosting with a vaccinia vector encoding the same EIAV Gag p15/p26, would induce robust Gag-specific CTL responses and protect horses against virulent EIAV challenge. Although this strategy elicited Gag-specific CTL in the majority of vaccinates, it was not protective. Unexpectedly, enhancement of viral load and clinical disease was observed, which was associated with the presence of vaccine-induced CTL.

Materials and Methods

Horses

Two yearling Arabian horses (A2197 and A2203), and ten mixed breed ponies (H654, H657, H658, H664, H671, H677, H681, H63, H684, H686) ranging from one to three years of age were used in this study (Tables 1 and 2). All had the equine leukocyte antigen (ELA)-A1 haplotype as determined serologically by lymphocyte microcytotoxicity [56–58] using reagents kindly provided by Dr. Ernest Bailey (University of Kentucky, Lexington, KY). Both A2197 and A2203 inherited the ELA-A1 haplotype from their common sire A2152. This stallion has the ELA-A1-associated 7-6 MHC class I allele as determined by RT-PCR [59–61], and thus A2197 and A2203 had the 7-6 allele by descent. The 7-6 molecule presents the Gag-GW12 CTL epitope [34], which is contained within Gag p15 [34]. In addition, Gag-GW12-specific CD8⁺ T cells are identified by the 7-6/Gag-GW12 tetramer [31]. All ten ponies inherited the ELA-A1 haplotype from their common sire H600 [62], which did not have the 7-6 allele. The 7-6 allele was therefore absent by descent in all ten ponies. For each horse and pony, percutaneous kidney biopsies were performed and equine kidney (EK) cells were established in cell culture for use as targets in CTL assays [29,33,34]. All experiments involving horses and ponies were approved by the Washington State University Institutional Animal Care and Use Committee.

Construction of the equine IL-2/IgG plasmid

Construction of the equine IL-2/IgG plasmid was performed as described [51] with modifications. The plasmid pEQU-SPORT2 [63] was used as the source of equine IL-2 cDNA, and the plasmid IGHG1 [64] was used as the source of equine C γ 1 cDNA. The primers used to amplify IL-2 cDNA added a 5' BamHI site for insertion into the multiple cloning site of the VR-1055 eukaryotic expression vector (Vical, San Diego, CA), a Kozak consensus for optimum eukaryotic expression, and a 3' Ssp I site for ligation to C γ 1. The primers used to amplify C γ 1 cDNA inserted a 5' Ssp I site for ligation to IL-2, a glutamine and a glutamic acid to resemble the hydrophilic-large hydrophobic-hydrophilic-hydrophilic pattern of amino acids commonly present at the carboxy end of VDJ segments [51], and a 3' BamHI site for insertion into VR-1055. After PCR amplification of the IL-2 and C γ 1 cDNA, the 2 genes were digested with BamHI and SspI, agarose gel purified, and inserted into VR-1055 by 3-way ligation. The resulting plasmid was designated VR-IL2/IgG.

To verify expression, COS-7L cells were transfected with VR-IL2/IgG using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to manufacturers instructions. Briefly, 60 μ g plasmid DNA was added to 2.8 ml Optimem serum free medium, which was then combined with 2.8 ml Optimem to which 198 μ l Lipofectamine 2000 had been added. This mixture was incubated at room temperature for 20 minutes, then added to COS-7L cells in 175 cm² flasks containing 10 ml of DMEM + 2.5% FBS and incubated overnight at 37°C with 5% CO₂. The transfection media was then removed and the monolayer rinsed with VP-SFM serum free media. Twenty ml VP-SFM + L-glutamine was then added and the flasks incubated another 72 hours. Media from the transfected cells was concentrated and used for immunoblotting under non-reducing and reducing conditions with goat anti-horse IgG(H+L) HRP antibody (KPL, Gaithersburg, MD) for detection of the equine IL2/IgG fusion protein and equine IgG. Biological IL-2 activity in the VR-IL2/IgG transfected COS-7L cell media was determined as described [65].

Construction of the codon-optimized EIAV Gag p15 and p15/p26 plasmid

The EIAV gag p15 and p26 sequences (EIAV_{WSU5} nt 475–1551; GenBank accession no. AF247394) were codon-optimized for Equidae genes. Equidae and EIAV codon usage was obtained from <http://www.kazusa.or.jp/codon/> and the gag p15 and p26 genes were optimized

and synthesized commercially by GenScript Corp. (Piscataway, NJ). The optimized *gag* genes plus a Kozak consensus sequence for optimum eukaryotic expression were inserted into the multiple cloning site of VR-1055 (Vical). The first plasmid made contained the p15 sequence only, and was designated VR-p15. This plasmid was used in a pilot experiment involving horses A2197 and A2203. Subsequently, VR-p15/p26 (which contained both *gag* p15 and p26) was constructed. Equine kidney (EK) cells were transfected with VR-p15 or VR-p15/p26 using Lipofectamine 2000 (Invitrogen), and immunoblotting confirmed Gag p15 (for VR-p15) or Gag p15 and p26 (for VR-p15/p26) protein expression in transfected cell lysate using immune serum (containing antibodies against EIAV Gag p15) and mAb 30-6A1, against EIAV Gag p26 [66]. Large preparations of endotoxin-free VR-p15 and VR-p15/p26 were obtained using a Plasmid Giga Kit (Qiagen Inc., Valenica, CA).

Construction of the codon-optimized EIAV Gag p15/p26 vaccinia vector

Codon-optimized *gag* p15/p26 was inserted between the vaccinia synthetic promoter and early transcription termination signal of the plasmid transfer vector pRB21 [67] to make pRBp15/26. Advantages of this vaccinia vector system include simplified and reliable selection of plaque forming units, and high expression of inserted genes afforded by the strong synthetic early/late promoter [67]. Following infection with the non-plaque forming vRB12 vaccinia mutant and transfection with pRBp15/26, plaques of recombinant vaccinia virus (designated vac-p15/p26) were isolated and plaque purified three times to eliminate possible vRB12 mutant virus contaminants. In addition, empty pRB21 vaccinia recombinants were also made to use as negative controls. Immunoblotting was used to detect Gag p15 and p26 protein expression in vac-p15/p26 infected BSC-1 cell lysates as above. To purify and expand the recombinant virus stock for injection into horses, plaque purified recombinant virus was amplified initially in BSC-1 cells then further amplified in Hela cells to obtain sufficient virus stocks for purification on a sucrose step gradient.

Pilot immunizations with VR-p15, VR-p15/p26, VR-IL2/IgG, and the previously constructed vGag/PR recombinant vaccinia virus vector in horses A2197 and A2203

In an initial pilot experiment, horses A2197 and A2203 were injected intramuscularly (IM) with 5 mg VR-p15 and 5 mg VR-IL2/IgG, four times at four week intervals (Table 1; Fig. 1a). For injections, 5 mg of each plasmid was first diluted in sterile saline, then combined (total volume 2.8 ml) and injected into the left pectoral muscle. Three months after the fourth injection, both horses were then injected with 5 mg VR-p15/p26 and 5 mg VR-IL2/IgG, four times at two week intervals. The injection procedure was exactly as before. Two weeks after the last plasmid injection, both horses were boosted intradermally (ID) with the previously constructed vGag/PR recombinant vaccinia virus, which contains the EIAV *gag* and 5' *pol* genes [66]. This was done twice, four weeks apart. For each vaccinia inoculation, a total dose of 4×10^8 plaque forming units (PFU) vGag/PR was divided and injected ID with scarification in two sites on the neck (0.5 ml/site).

Immunizations with VR-p15/p26, VR-IL2/IgG, and the vac-p15/p26 recombinant vaccinia virus vector in ponies

The ten ponies were age-matched and split into two groups of five controls (H657, H658, H677, H683, H686) and five vaccinates (H654, H664, H671, H681, H684) (Table 2; Fig. 1b). The vaccinates received ID injections of 2.5 mg VR-p15/p26 and 2.5 mg VR-IL2/IgG, four times at 2 week intervals. For each injection, 2.5 mg of each plasmid was first diluted in sterile saline, then combined (total volume 1 ml) and injected ID in ten sites on the neck (100 μ l/site). The controls were injected the same way, except that 2.5 mg empty VR-1055 and 2.5 mg VR-IL2/IgG were used. One month after the fourth injection, the vaccinates received three more injections of VR-p15/p26 without VR-IL2/IgG, at two week intervals. For each injection, 2.5

mg VR-p15/p26 was diluted in 1 ml sterile saline and injected ID in ten sites on the neck (100 µl/site) as before. The controls received empty VR-1055 in the same way. Two weeks after the last plasmid injection, the vaccinates were boosted ID with the vac-p15/p26 recombinant vaccinia virus, twice, four weeks apart. For each vaccinia inoculation, a total dose of 4×10^8 PFU vac-p15/p26 was divided and injected ID with scarification in two sites on the neck (0.5 ml/site). The controls were injected the same way, but received the empty vRB21 vaccinia vector.

CTL assays

CTL assays were performed as described [31,34,36] with modifications. Briefly, peripheral blood mononuclear cells (PBMC) were isolated every two weeks for direct detection of CTL activity, and for stimulation with peptide-pulsed monocytes. For peptide stimulations, seven peptide pools were used, each containing 10–14 synthetic peptides covering the entire EIAV_{WSU5} Gag p15 and p26 proteins [36]. These peptide pools contain the four previously described CTL epitope clusters 1–4 [36]. Epitope cluster (EC) 1 is contained in peptide pool 1, EC2 is contained in pool 2, EC3 is contained in pools 6 and 7, and EC4 is contained in pool 7 [36]. Peptide pools (final concentration of each peptide was 10^3 nM) and PBMC were incubated for 2 h at 37°C with occasional mixing before centrifugation at $250 \times g$ for 10 min. PBMC were resuspended to 2×10^6 /ml in RPMI 1640 medium with 10% FBS, 20 mM HEPES, 10 µg/ml gentamicin, and 10 µM 2-ME. One ml of resuspended cells was added to each well of a 24-well plate and incubated for 1 wk at 37°C before use in CTL assays. CTL activity was measured in freshly isolated and stimulated PBMC with a 17-h ^{51}Cr release assay using autologous EK target cells obtained by biopsy. Target cells were pulsed with the same peptide pools used for stimulation, at a final concentration of 10^4 nM for each peptide. The formula, % specific lysis = $[(E-S)/(M-S)] \times 100$, was used, where E is the mean of three test wells, S is the mean spontaneous release from three target cell wells without effector cells, and M is the mean maximal release from three target cell wells with 2% Triton X-100 in distilled water. The E:T cell ratio was 50:1, and each well contained ~30,000 target cells. Only assays with a spontaneous target cell lysis of <30% were used. The SE (standard error) of percent specific lysis was calculated using a formula that accounts for the variability of E, S, and M [68]. Significant lysis was defined as the percent specific lysis of peptide-pulsed target cells that was >10% and also >3 SE above the nonpulsed target cells.

Tetramer analysis for Gag-GW12-specific CD8⁺ T lymphocytes in A2197 and A2203

Tetramer analysis was performed only in horses A2197 and A2203 because these horses were the only two with the 7-6 allele. The 7-6/Gag-GW12 tetramer was used to identify Gag-GW12-specific CD8⁺ T lymphocytes in freshly isolated and stimulated PBMC as described [31] with modifications. Briefly, the murine anti-equine CD3 monoclonal antibody F6G [69,70] and the murine anti-equine CD8 monoclonal antibody HT14A [71] were directly labeled with Alexa Fluor 647 and 488 dyes, respectively, using the Alexa Fluor Monoclonal Antibody Labeling Kit (Invitrogen). Gag-GW12 peptide stimulations were done as described above using 10^3 nM Gag-GW12. Peptide-stimulated PBMC were stained first with PE-conjugated 7-6/Gag-GW12 tetramer for 30 min at 37°C, washed, then directly labeled F6G and HT14A were added for 15 min at 4°C. For analysis of stimulated PBMC, propidium iodide staining was performed to assess cell viability. Three-color (or four-color for stimulated PBMC) analysis was performed on live CD3-gated lymphocytes using a FACSsort flow cytometer (Becton Dickinson, Franklin Lakes, NJ), with Cell Quest and Paint-A-Gate Pro software.

Detection of EIAV-specific antibody responses

Serum antibodies against EIAV Gag p26 were detected using the Equine Infectious Anemia Virus Antibody ELISA Test Kit (VMRD, Inc., Pullman, WA), according to manufacturer's instructions.

Immunologic and virologic monitoring following EIAV challenge

The two horses used in the initial pilot experiment (A2197 and A2203) were not challenged. However, all ten ponies used in the subsequent experiment were inoculated intravenously with 300 TCID₅₀ EIAV_{PV} [62,72,73], four weeks after the last vaccinia vector boost. Following EIAV challenge, rectal temperatures and clinical status were recorded daily, and CBC with platelet counts were performed on whole blood collected from the ten ponies every three days. Platelet counts (platelets/ μ l) at least three SD below the pre-challenge mean were considered thrombocytopenic for each pony. Plasma and serum were collected and stored at -80°C every three days. Viral load was determined by extracting plasma viral RNA and performing real-time quantitative RT-PCR as described [31]. Whole blood was collected every two weeks for isolation of PBMC for CTL assays. To assess the possibility of Gag-specific CTL selection pressure, the EIAV *gag* p15 and p26 genes were amplified by RT-PCR from plasma viral RNA extracted from each pony during the initial viremic episode, cloned, and 20 clones sequenced as described [41].

To detect virus neutralizing antibodies in serum, a focal reduction assay was used [74], with slight modification. Briefly, serum was heat inactivated to destroy complement, serially diluted two-fold in DMEM, and incubated at 37°C for 1 h with 550 focus forming units (FFU) of EIAV_{PV}. Quadruplicate wells of EK cells in 12-well plates were inoculated with the virus-serum mixture, and the culture media was changed the following day. Cells were incubated for an additional 4 days, fixed with ice-cold methanol, and foci of EIAV_{PV}-infected cells were detected by immunocytochemistry and enumerated. Results for each pony were expressed as the serum neutralization titer, defined as the highest serum dilution that gave a 50% reduction in FFU as compared with pre-vaccination serum from the same pony.

Statistical analysis

A nonparametric Mann-Whitney test [75] was used for comparison of geometric mean viral load for each day tested, and of initial peak viral load between controls and vaccinates (or between ponies with vaccine-elicited CTL and those without), using a two-tailed significance level of $\alpha = 0.05$. For comparisons of percent days plasma viral load $> 10,000$ RNA copies/ml, and of percent days thrombocytopenic between controls and vaccinates (or between ponies with vaccine-elicited CTL and those without), a t test was used with a two-tailed significance level of $\alpha = 0.05$. A viral load value of $> 10,000$ RNA copies/ml was chosen for comparison because clinically apparent infection with fever and declining platelet counts associated with this viral load is consistently observed in EIAV-infected horses [31,34,65], and a viral load set point at this level distinguishes between EIAV progressor and nonprogressor horses [76]. Statistical analyses were performed using GraphPad InStat 3.06 and GraphPad Prism 5.01 (GraphPad Software, San Diego, CA).

Results

In vitro expression of the equine IL-2/IgG fusion protein by the VR-IL2/IgG plasmid vector

Expression of the VR-IL2/IgG plasmid was confirmed in two ways. First, COS-7L cells were transfected with VR-IL2/IgG and immunoblotting was used to detect the equine IL-2/IgG fusion protein in cell culture supernatant. Since the MW of equine IL-2 is 17 kDa [77] and that of an equine IgG heavy chain monomer is 53 kDa [78], the MW of the equine IL-2/IgG chimeric

homodimer was predicted to be 140 kDa. As expected, a 140 kDa band was confirmed by immunoblotting with goat anti-equine IgG(H+L) under non-reducing conditions (Fig. 2a). Bands of higher MW likely represented aggregates. After cleavage of the disulfide-linked heavy chains (each with an attached IL-2 molecule), the MW of the IL-2/IgG chimeric monomer was predicted to be 70 kDa. Expectedly, immunoblotting with goat anti-equine IgG (H+L) under reducing conditions produced a single 70 kDa band (Fig. 2a). Second, the IL-2 activity of the expressed IL-2/IgG chimeric molecule was confirmed in a functional assay. Equine PHA-activated lymphoblasts proliferated in a dose-dependent fashion after addition of VR-IL2/IgG-transfected cell culture supernatant (Fig. 2b). Thus, the VR-IL2/IgG plasmid resulted in expression of a fusion protein with the correct MW that contained equine IgG and had the functional characteristics of equine IL-2.

In vitro expression of EIAV Gag p15/p26 by plasmid and vaccinia vaccine vectors

To confirm expression of Gag p15 and p26 by the VR-p15 and VR-p15/p26 plasmids, EK cells were transfected with VR-p15 or VR-p15/p26 and cell lysates were reacted with immune serum containing antibodies against EIAV Gag p15, or with mAb 30-6A1 (against EIAV Gag p26) [66]. Immunoblotting detected the expected 15 kDa band in VR-p15 transfected cell lysate (Fig. 2c), while 55 and 26 kDa bands representing the Gag precursor and p26 proteins were detected in VR-p15/p26 transfected cell lysate (Fig. 2d). Expression of these same proteins by cells infected with the vac-p15/p26 recombinant vaccinia virus vector was similarly confirmed (data not shown).

To determine if codon optimization resulted in higher expression of Gag p26 in equine cells, equal numbers of EK cells were transfected with 60 µg of the codon-optimized VR-p15/p26 construct or 60 µg of a VR-1055 construct containing the native EIAV *gag* p15/p26 gene sequence. Immunoblotting performed as above confirmed that the codon optimized plasmid resulted in greater protein expression than the non codon-optimized plasmid (Fig. 2e).

CTL and antibody responses induced by vaccination

For the two horses used in the pilot experiment (A2197 and A2203), neither CTL activity nor tetramer positive CD8⁺ T cells were detected following the intramuscular VR-p15+VR-IL2/IgG and VR-p15/p26+VR-IL2/IgG plasmid injections. However, serum antibodies against Gag p26 were detected two weeks after the second VR-p15/p26+VR-IL2/IgG injection in A2197 and two weeks after the third VR-p15/p26+VR-IL2/IgG injection in A2203, confirming immunization (data not shown). Two weeks after the first vGag/PR vaccinia injection, CTL were detected in A2203 by ⁵¹Cr release assay, and the response increased two weeks after the second vGag/PR injection (Fig. 3a). Two weeks after the first and second vGag/PR injections, 31.4% and 35.0% of stimulated A2203 CD8⁺ T cells were 7-6/Gag-GW12 tetramer positive, respectively (Fig. 3b). Neither CTL activity nor 7-6/Gag-GW12 tetramer positive CD8⁺ T cells were detected in A2197. These results indicated that intramuscular injection of the VR-p15, VR-p15/p26, and VR-IL2/IgG plasmid DNA was unlikely to induce CTL responses in horses, but that induction of Gag-specific CTL with a vaccinia vector was feasible.

For the ponies, antibodies against Gag p26 were detected in all the vaccinates (but not the controls) after the second and third intradermal VR-p15/p26+VR-IL2/IgG plasmid injections, confirming immunization (data not shown). No CTL activity was detected following the four VR-p15/p26+VR-IL2/IgG plasmid injections (Figs. 4 and 5), except in vaccinated pony H681. This pony developed a transient low-level CTL response against Gag pool 5, detected two weeks after the third injection, and a transient low-level response against Gag pool 2, detected two weeks after the fifth injection (Fig. 4d). To rule out potential interference between the co-administered VR-p15/p26 and VR-IL2/IgG plasmids in the induction of CTL, the VR-p15/p26 plasmid was administered alone for an additional three intradermal injections. Again, CTL

responses were not detected (including pony H681). In contrast, Gag-specific CTL responses were detected in vaccinates H654 (Gag pool 1), H671 (Gag pools 1 and 2), and H681 (Gag pools 1 and 7) following the first and/or second vac-p15/p26 vaccinia injections (Fig. 4a,c,d). No Gag-specific CTL responses were detected prior to EIAV challenge in any of the control ponies (Fig. 5) or in vaccinates H664 and H684 (Fig. 4b,e). Immediately prior to EIAV challenge, Gag p26-specific serum antibody titers were highest in the vaccinates in which Gag-specific CTL were induced (1:256 in H654, H671, and H681), and lower in the other two vaccinates (1:64 in H664 and H684). No Gag p26-specific antibodies were detected in any of the controls (Table 3).

Plasma viremia, clinical disease, and CTL and neutralizing antibody responses following virulent EIAV challenge

All ponies were challenged with EIAV_{PV} four weeks after the last vaccinia vector boost. Platelet counts and plasma viral loads were followed for 552 days post EIAV inoculation (DPI). All ponies became viremic and thrombocytopenic within the first month after challenge (Figs. 6 and 7). Vaccinate H664 (Fig. 6b), and controls H657 (Fig. 7a) and H686 (Fig. 7e) were humanely euthanized due to acute disease during the first two months after challenge. Initial analysis of viral loads for ponies in each group suggested that not only did the vaccine fail to induce protection, but that it resulted in enhancement of viral replication (Fig. 8a). The overall geometric mean viral load for each day was higher in the vaccinates compared to the controls, and the difference was highly significant (Fig. 8b). Likewise, the initial peak viral load was higher in the vaccinates (Fig. 8c). However, there was no difference in the initial peak viral load in ponies with vaccine-induced Gag-specific CTL (H654, H671, H681) as compared to that in ponies without vaccine-induced Gag-specific CTL (Fig. 8d). The vaccinates had a higher percentage of days with significant plasma viremia (> 10,000 viral RNA copies/ml) than the controls (Fig. 8e), which was associated with the presence of vaccine-induced Gag-specific CTL (Fig. 8f). In addition, clinical disease was more severe in the vaccinates, which had a higher percentage of thrombocytopenic days than the controls (Fig. 8g). Similar to the percentage of viremic days, the higher percentage of thrombocytopenic days was associated with the presence of vaccine-induced Gag-specific CTL (Fig. 8f). Finally, because vaccinate H664 and controls H657 and H686 were lost early in the study, data for only the first 30–47 DPI could be included in the above analyses for these ponies. To ensure this did not lead to spurious conclusions, the above analyses were repeated after excluding these three ponies entirely, and the same results were obtained (data not shown).

In the vaccinates with vaccine-induced Gag-specific CTL (H654, H671, and H681), CTL directed against Gag pools 1 and/or 2 were detected one week post EIAV challenge (Fig. 4a,c,d). In vaccinates H664 and H684, which had no vaccine-induced Gag-specific CTL, Gag-specific CTL were not detected until three and six weeks post EIAV challenge, respectively (Fig. 4b,e). These CTL were directed against Gag pools 1 (H664) and 3 (H684). Among the controls, Gag-specific CTL were not detected at any time point in H657, and were not detected in the other control ponies until three to six weeks post EIAV challenge (Fig. 5). These CTL recognized Gag pools 1 and 7 (H658), pool 3 (H677), pools 1, 6, and 7 (H683), and pools 2 and 6 (H686).

To evaluate the possibility that Gag-specific CTL escape variants contributed to the lack of viral control in the vaccinates, *gag* p15/p26 clones from plasma viral RNA were sequenced from each vaccinate and control during the initial viremic episode. For both the vaccinates and controls, the majority of clones (71% and 73%, respectively) had no amino acid changes within the four previously defined epitope cluster regions of Gag p15 and p26 [36], making it highly unlikely that vaccine-elicited CTL resulted in escape variants in the vaccinates (data not shown).

Serum neutralizing antibodies against EIAV_{PV} were not detected in either group following the vaccine regimen and prior to EIAV_{PV} challenge. Neutralizing antibodies were first detected 64 days post EIAV_{PV} inoculation (DPI) in the surviving ponies of both groups, and titers ranged from 1:4 to 1:8 (Table 4). By 83 DPI, neutralizing titers increased two-fold in three of the four surviving vaccinates, and four-fold in one of the three surviving controls. No increase in neutralizing titer was observed in the other remaining vaccinate and two controls. Overall, there appeared to be no clear differences between vaccinates and controls with respect to the appearance and titer of post-challenge neutralizing antibodies.

Discussion

Horses infected with EIAV provide a unique opportunity to dissect the correlates of CTL-mediated protection against lentivirus infection and disease. Moreover, since horses are a difficult species in which to elicit CTL using DNA-based methods [54], they constitute a rigorous outbred large animal model to test CTL induction strategies. The purpose of this study was to determine if an IL-2/IgG-augmented *gag* p15/p26 DNA prime-vaccinia vector boost vaccine regimen could elicit Gag-specific CTL that would protect horses against virulent EIAV challenge in the absence of neutralizing antibody. For the first time, we have shown that this regimen can elicit Gag-specific CTL in horses, but that the DNA priming component, which included co-administration of the IL-2/IgG plasmid, was not efficient. Despite the induction of Gag-specific CTL, this vaccine regimen was not protective against EIAV challenge. On the contrary, we unexpectedly observed higher viral loads and more severe clinical disease in the vaccinates, which were associated with the presence of vaccine-induced Gag-specific CTL. To our knowledge, this is the first description of such lentivirus enhancement associated with a DNA prime-viral vector boost T cell vaccine lacking envelope immunogens.

Although plasmid DNA administered either IM or ID elicited serum antibodies, neither route resulted in a detectable/sustained CTL response despite up to eight injections two to four weeks apart. However, CTL were observed following the vaccinia vector boost in both cases. Similar results were reported with a DNA prime-recombinant modified vaccinia Ankara (rMVA) boost regimen in horses that induced protective antibody and cellular immune responses against equine influenza virus [79]. In that study, neither antibody nor cellular (as measured by lymphocyte proliferation and IFN- γ expression) responses were observed until after the rMVA boost. In fact, rMVA vaccination was efficacious without the DNA prime [79]. Likewise, equine herpesvirus-1-specific CTL can be elicited in horses by vaccination with a vaccinia vector without a DNA prime, although the induced CTL response is not protective [55]. Thus, vaccinia-based vectors can elicit CTL and cellular responses in horses without DNA priming. However, it is currently unknown whether or not more robust and protective CTL responses would be induced in the horse if efficient DNA priming occurred prior to administration of recombinant viral vectors.

In contrast to the results obtained in the current study, we previously elicited Gag-specific CTL and tetramer positive CD8⁺ T cells in horses with the same codon-optimized *gag* p15/p26 plasmid [54]. The naked plasmid DNA was administered four times at 2–4 week intervals, and each 5 mg dose was divided and administered by ID and intratracheal (IT) routes [54]. It is possible that the IT component contributed to the induction of CTL in that study by providing additional uptake at inductive sites in the upper and lower respiratory tract, or in the pharynx. Mucosa-associated lymphoid tissue (MALT) is present throughout the equine respiratory tract, including the nasopharynx, trachea, bronchi, and bronchioles [80–82]. However, work in mice indicates that intranasal (IN) delivery of naked DNA is not an effective strategy for inducing systemic CTL responses, and that plasmid DNA must be incorporated into cationic liposomes or targeted to mucosal inductive sites such as M cells to elicit robust systemic CTL responses [83,84]. Since results in mice frequently do not correlate with those in larger animals, further

investigation is needed to determine if similar modifications to plasmids administered via the respiratory tract are necessary to elicit systemic CTL responses in horses, and whether or not a respiratory route alone is sufficient to elicit such responses. Thus, the specific contribution of the IT component to the induction of CTL observed in our previous study is difficult to discern.

Although work in mice indicates that administration of similar IL-2/Ig or IL-2 plasmids at the same time as HIV-1 DNA vaccines enhances antigen-specific antibody, cell-mediated, and CTL responses [85,86], the efficiency of DNA priming in the current study could have been affected by the timing of IL-2/IgG plasmid delivery. In macaques, plasmid vaccination with HIV-1 89.6P DNA and SIV mac239 DNA (5 mg each, IM) on weeks 0, 4, and 8 elicits SIV Gag-specific CTL, and the response is augmented when an IL-2/Ig plasmid (5 mg, IM) is administered two days after the week 2 immunization, and again two days after the week 4 immunization [50]. This regimen is protective against SHIV 89.6P challenge [53]. In mice, antibody and CTL responses elicited by an HIV-1 gp120 DNA vaccine are augmented when an IL-2/Ig plasmid is administered two days after the gp120 plasmid, but antibody responses are suppressed when the IL-2/Ig plasmid is administered either five days before or concurrently with the gp120 plasmid, and CTL responses are decreased when the IL-2/Ig plasmid is administered five days before [87]. In contrast, non-specific splenocyte proliferation increases when the IL-2/Ig plasmid is administered before or with the vaccine plasmid [87]. It was concluded that IL-2/Ig exposure to a naïve immune system results in nonspecific cellular activation above which specific immune responses are elicited poorly, and that IL-2/Ig exposure shortly after priming amplifies the immune response [87]. Based on this work in mice, the co-administration of the VR-IL2/IgG and VR-p15/p26 plasmids in the current study could have inhibited CTL induction. Given the different outcomes in other model systems, it remains to be determined whether or not co-administration of an IL-2/IgG plasmid with a DNA vaccine inhibits antigen-specific immune responses in the horse.

Although the current vaccine regimen elicited Gag-specific CTL in three of the five vaccinates, these vaccinates were not protected against challenge. Possible conclusions are that Gag-specific CTL alone are not sufficient for protection against EIAV, that the elicited CTL response was neither broad nor robust enough to be protective, or that vaccine-elicited Gag-specific CTL escape variants arose after challenge. Although viral RNA *gag* sequencing made the latter conclusion unlikely, the former possibilities require further investigation. The requirements for protective CTL responses against lentiviral infection, and whether CTL can be protective in the absence of neutralizing antibody, are still not known. Important knowledge gaps continue to include the specificity and number of epitopes that must be recognized, the functional avidity of responding CTL, as well as the quantitative magnitude of the CTL response. Our previous work suggests that Gag-specific CTL are important in EIAV control since the majority of inapparent carriers have CTL recognizing epitopes in Gag [33], Gag CTL frequency can correlate with control of viremia [31], CTL epitope clusters occur in Gag [36], and high avidity CTL recognizing Gag epitope clusters correlate with lack of clinical disease [41]. In the current study, vaccine elicited CTL recognized Gag pools 1, 2, and 7, which contain epitopes within three of the four previously identified epitope clusters, EC1, 2, and 4, respectively [36]. Despite the fact that multiple Gag epitopes were targeted, the levels of specific lysis were modest, and a vaccine regimen protective against EIAV challenge will most likely need to elicit a broader and more robust CTL response. A similar conclusion could be drawn based on the failed STEP trial, in which the human vaccinees mounted relatively weak T cell responses against a total of only three epitopes in the Gag, Pol, and Nef immunogens [17].

Importantly, not only did the current vaccine regimen fail to protect against EIAV challenge, but it resulted in enhanced viral load and clinical disease. Although vaccine-induced

enhancement of infection has been observed in EIAV [73,88] and likely in HIV-1 [89], envelope immunogens were used, and antibody-dependent enhancement (ADE) was the probable cause [89–91]. Antibodies that enhance lentivirus infection have been demonstrated in serum from HIV-1-infected humans [89,92] and chimpanzees [93], and in plasma from SIV-infected macaques [94]. For HIV-1 and SIV, ADE can be complement-dependent or independent [92–94]. Complement-independent ADE of HIV-1 infection occurs via Fc receptor-mediated entry into monocytes [95], which is the likely mechanism for ADE of EIAV infection since complement is not required [91]. Despite the documented importance of ADE in lentivirus infections (including EIAV), the vaccine in the current study did not include envelope immunogens. Although relatively weak Gag p26-specific antibody responses were elicited, capsid-specific antibodies are not known to contribute to ADE in lentivirus infections. Thus, described mechanisms of ADE did not contribute to the increased viral load and more severe clinical disease observed in the vaccinates.

A possibly related observation was recently made in cats following vaccination with canine adenovirus vectors encoding feline immunodeficiency virus (FIV) Gag, Env, or a GFP control [96]. Although Env-specific antibodies were not detected following vaccination with the Env vector, Gag-specific antibodies were detected in three of six cats following vaccination with the Gag vector. Neither vaccine protected cats against FIV challenge. While the time to initial peak viral load appeared to be the same between groups, plasma virus could be detected one to two weeks earlier in five of the six cats receiving the Gag vector, as compared to cats vaccinated with the Env or GFP control vectors [96]. In contrast to the present study, there were no differences between groups in the magnitude of the initial peak viral load or in the viral loads once they stabilized, and no differences in clinical disease were reported. Although the authors speculate that cellular responses were involved in the enhancement of early viral replication in the Gag-vaccinated cats, cellular immune responses elicited by the vaccine or by infection were not measured.

In the present study, enhanced viral load and clinical disease correlated with the presence of vaccine-elicited Gag-specific CTL. However, it is not thought that Gag-specific CTL directly resulted in increased viral replication. Instead, the induction of these non-protective CTL could have been an indicator of successful T cell immunization, and that along with Gag-specific CTL, Gag-specific T regulatory cells (Treg) might have been elicited. The observation that the three vaccinates in which Gag-specific CTL were induced (H654, H671, and H681) also had higher Gag p26 serum antibody titers (Table 3) suggested that overall immunization was most efficient in these ponies, increasing the likelihood of Treg induction. In mice and humans, Treg are CD4⁺ T cells that constitutively express the high affinity IL-2 receptor α -chain (CD25), and the transcription factor Foxp3 [97]. The function of Treg are to suppress immune responses in a contact-dependent manner in response to T cell receptor (TCR) stimulation [97]. Although very little is known regarding CD4⁺CD25⁺Foxp3⁺ Treg in the horse, TCR diversity of CD4⁺/CD25⁺ Treg in humans is broad and similar to that in CD4⁺/CD25⁺ T cells, suggesting that the same antigen could elicit either an effector T cell response or a Treg response [97,98]. In the current vaccine regimen, the IL-2/IgG plasmid was administered concurrently with the Gag p15/p26 plasmid. As noted above, administration of an IL-2/Ig plasmid before or at the same time as a vaccine plasmid in mice leads to diminished antigen-specific immune responses, but increased non-specific splenocyte proliferation [87]. Moreover, administration of a similar IL-2/Ig plasmid in macaques results in a dramatic increase in CD25⁺ T cells [50]. In addition, low-dose IL-2 therapy in HIV-1-infected patients results in expansion and activation of Treg [99]. Taken together, these data raise the possibility that the co-administration of the IL-2/IgG plasmid with the gag p15/p26 plasmid in the current study could have elicited Gag-specific Treg that suppressed potentially protective Gag-specific immune responses in the vaccinates. Reagents and methods to characterize antigen-specific Treg responses in the horse are just now

becoming available, and dissecting the possible role of Treg in the vaccine enhancement observed in the present study is a focus of ongoing investigation.

Although the co-administration of the IL-2/IgG plasmid in the present study could have played a role in suppressing CTL responses, it is also possible that the repeated and prolonged administration of the *gag* p15/p26 plasmids could have resulted in tolerance rather than immunity. Persistent viral infection resulting in chronic antigen exposure can lead to exhaustion of CD8+ T cell responses in LCMV-infected mice and in HIV-1-infected humans [100–103]. Additional controls, including a *gag* p15/p26 plasmid prime-vaccinia boost group (no IL-2/IgG plasmid), and a vaccinia alone group (no *gag* p15/p26 plasmid prime, no IL-2/IgG plasmid) would be required to determine if the viral load and clinical disease enhancement observed in the present study was due to the *gag* p15/p26 plasmid injection regimen, or to the administration of the IL-2/IgG plasmid, or to a combination of both.

In summary, horses constitute a rigorous outbred large animal model species in which to test DNA-based T cell vaccine strategies against lentivirus infection. Our results indicate that EIAV Gag-specific CTL can be elicited with a DNA prime-vaccinia vector boost vaccine regimen. However, the DNA priming component was inefficient despite co-administration of an IL-2/IgG plasmid. Interestingly, this strategy has both augmented and suppressed DNA vaccine-induced immune responses in other model systems. Further work is needed to determine the optimal dose, route, and timing of IL-2/IgG and vaccine plasmids to efficiently prime and augment CTL responses in the horse. Although Gag-specific CTL were eventually elicited, they were not protective against virulent EIAV challenge, and surprisingly, enhanced viral load and clinical disease were observed in the vaccinates. This is the first report of such lentivirus enhancement associated with a T cell vaccine not containing envelope immunogens, and as such, provides important implications for lentivirus T cell vaccine design. Specifically, whether or not the current vaccine regimen resulted in the activation of Treg that ultimately suppressed lentivirus-specific immune responses leading to enhanced disease will be critical to determine.

Acknowledgments

The important technical assistance of Emma Karel and Lori Fuller is acknowledged. This work was supported in part by U.S. Public Health Service, National Institutes of Health grants AI073101, AI067125, and AI60395.

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Figure 1.

a. Timeline for the pilot vaccine experiment in horses A2197 and A2203. Injections of the different plasmids and the vaccinia vector were timed as indicated by the open circles, squares, and triangles. Arrows indicate times when CTL activity was assayed. IM, intramuscular. ID, intradermal. **b.** Timeline for the vaccine experiment in ten ponies. Annotations are similar to those in 1a, with the time of EIAV challenge indicated by the black diamond. IV, intravenous.

Figure 2.

a. Immunoblot of VR-IL2/IgG-transfected COS7L cell culture supernatant reacted with goat anti-equine IgG(H+L). VR-IL2/IgG reduced (lane 1), VR-1055 empty vector reduced (lane 2), VR-IL2/IgG non-reduced (lane 3), and VR-1055 non-reduced (lane 4). **b.** Biological IL-2 activity of VR-IL2/IgG-transfected and VR-1055-transfected COS-7L cell media. Equivalent concentrations of each were added to 7-day cultures of PHA-stimulated equine PBMC in triplicate, and ^3H thymidine uptake was determined after 2 days. CPM, counts per minute. Error bars are SD. **c.** Immunoblot of VR-p15-transfected EK cell lysate using 1:100 dilution of equine immune serum. Normal EK cells (lane 1), VR-p15 (lane 2), VR-1055 (lane 3), and 1 µg EIAV_{WSU5} positive control (lane 4). **d.** Immunoblot of VR-p15/p26-transfected EK cell lysate using mAb 30-6A1 against Gag p26. Normal EK cells (lane 1), VR-p15/p26 (lane 2), VR-1055 (lane 3), and 1 µg EIAV_{WSU5} positive control (lane 4). **e.** Immunoblot comparing VR-p15/p26 expression vs non codon-optimized VR-p15/p26. Transfected EK cell lysate was reacted with mAb 30-6A1 against Gag p26. VR-p15/p26 (lane 1), non codon-optimized VR-p15/p26 (lanes 2 and 3), and VR-1055 (lane 4).

Figure 3.

a. Immunization with the vaccinia vector vGag/PR induced Gag-specific CTL in horse A2203. PBMC obtained at the indicated time-points were stimulated for one week with Gag peptide pools 1–7 and CTL activity determined on Gag peptide pool-pulsed autologous EK targets. Error bars are SE. E:T ratio, 50:1. **b.** Immunization with vGag/PR induced Gag-GW12-specific CD8⁺ T cells. PBMC obtained from vaccinated horse A2203 at the indicated time-points were stimulated for one week with the Gag-GW12 peptide, then labeled with the 7-6/Gag-GW12 tetramer. Percent tetramer positive CD8⁺ cells were determined on CD3⁺ gated lymphocytes using flow cytometry. Left panels are no tetramer controls.

Figure 4.
a–e. Gag-specific CTL responses in vaccinates H654, H664, H671, H681, and H684. PBMC obtained at the indicated time-points were stimulated for one week with Gag peptide pools 1–7 and CTL activity determined on Gag peptide pool-pulsed autologous EK targets. Background specific lysis of autologous EK targets not pulsed with peptide was subtracted from the values shown. Error bars are SE. E:T ratio, 50:1. Specific lysis >10% (horizontal dotted line) and also >3 SE above the nonpulsed target cells was considered significant. Time points associated with plasmid injections, vaccinia vector injections, and EIAV challenge are indicated.

Figure 5.
a–e. Gag-specific CTL responses in controls H657, H658, H677, H683, and H686. Annotations are the same as for figure 3.

Figure 6.

a–e. Peripheral blood platelet counts (—■) and plasma viral loads (—●) for vaccinees H654, H664, H671, H681, and H684. Vaccinee H664 was humanely euthanized on DPI 40 due to acute clinical disease.

Figure 7.
a–e. Peripheral blood platelet counts (—■) and plasma viral loads (—●) for controls H657, H658, H677, H683, and H686. Controls H657 and H686 were humanely euthanized on DPI 30 and DPI 44, respectively, due to acute clinical disease.

Figure 8.

Viral load and clinical disease enhancement occurred in vaccinates. **a.** Mean log plasma viral loads on each day tested for controls (thin red line) and vaccinates (thin blue line). Error bars are standard error. The bold lines are 2nd order smoothed curves for controls (red) and vaccinates (blue). **b.** Geometric mean plasma viral loads on each day tested for controls and vaccinates. Horizontal lines are medians. **c.** Initial peak plasma viral load for controls and vaccinates. Horizontal lines are geometric means. **d.** Initial peak plasma viral loads for ponies that had vaccine-elicited CTL compared with those that did not. Horizontal lines are geometric means. **e.** Percent days plasma viral load exceeded 10,000 RNA copies/ml for controls and vaccinates. Horizontal lines are means. **f.** Percent days plasma viral load exceeded 10,000 RNA copies/ml for ponies that had vaccine-elicited CTL compared with those that did not. Horizontal lines are means. **g.** Percent days platelet counts were at least 3 SD below the pre-challenge

mean for controls and vaccinates. Horizontal lines are means. **h.** Percent days platelet counts were at least 3 SD below the pre-challenge mean for ponies that had vaccine-elicited CTL compared with those that did not. Horizontal lines are means.

Table 1

Horse description and immunization protocol for initial pilot experiment

Horse	Age (yrs)	ELA-A Haplotype ^d	Group	Weeks 0, 4, 8, 12 ^b	Immunization Protocol	
					Weeks 24, 26, 28, 30 ^b	Weeks 32 and 34 ^c
A2197	1	A1/A4	Pilot	VR-p15+VR-IL2/IgG	VR-p15/p26+VR-IL2/IgG	vGag/PR
A2203	1	A1	Pilot	VR-p15+VR-IL2/IgG	VR-p15/p26+VR-IL2/IgG	vGag/PR

^a ELA-A haplotypes were determined serologically by lymphocyte microcytotoxicity. It was not known whether horses with one haplotype were homozygous or heterozygous with a haplotype not recognized by available antisera.

^b For each plasmid, 5 mg of DNA was injected IM.

^c 4×10^8 PFU vaccinia vector was injected ID.

Table 2

Description of ponies and experimental protocol for vaccine study

Pony	Age (yrs)	ELA-A Haplotype ^a	Group	Weeks 0, 2, 4, 6 ^b	Immunization Protocol		
					Weeks 10, 12, 14 ^b	Weeks 16 and 18 ^c	Week 22 ^d
H654	3	A1/A5	Vaccinate	VR-p15/p26+VR-IL2/IgG	VR-p15/p26	vac-p15/p26	EIAV challenge
H664	3	A1/A5	Vaccinate	VR-p15/p26+VR-IL2/IgG	VR-p15/p26	vac-p15/p26	EIAV challenge
H671	2	A1/A3	Vaccinate	VR-p15/p26+VR-IL2/IgG	VR-p15/p26	vac-p15/p26	EIAV challenge
H681	1	A1/A10	Vaccinate	VR-p15/p26+VR-IL2/IgG	VR-p15/p26	vac-p15/p26	EIAV challenge
H684	1	A1/w11	Vaccinate	VR-p15/p26+VR-IL2/IgG	VR-p15/p26	vac-p15/p26	EIAV challenge
H657	3	A1	Control	VR-1055+VR-IL2/IgG	VR-1055	vRB21	EIAV challenge
H658	3	A1/A5	Control	VR-1055+VR-IL2/IgG	VR-1055	vRB21	EIAV challenge
H677	2	A1/w11	Control	VR-1055+VR-IL2/IgG	VR-1055	vRB21	EIAV challenge
H683	1	A1/A5	Control	VR-1055+VR-IL2/IgG	VR-1055	vRB21	EIAV challenge
H686	1	A1	Control	VR-1055+VR-IL2/IgG	VR-1055	vRB21	EIAV challenge

^a ELA-A haplotypes were determined serologically by lymphocyte microcytotoxicity. It was not known whether horses with one haplotype were homozygous or heterozygous with a haplotype not recognized by available antisera.

^b For each plasmid, 2.5 mg of DNA was injected ID. VR-1055, empty plasmid control vector.

^c 4×10^8 PFU vaccinia vector was injected ID. vRB21, empty vaccinia control vector.

^d All ponies were inoculated IV with 300 TCID50 EIAVPV

Table 3

Gag p26 serum antibody titers immediately prior to EIAV challenge

Pony	Serum Antibody Titer
Vaccinates	
H654	1:256
H664	1:64
H671	1:256
H681	1:256
H684	1:64
Controls	
H657	neg
H658	neg
H677	neg
H683	neg
H686	neg

Table 4

Serum neutralizing antibody responses after EIAV Challenge

Pony	Pre	29	Days Post EIAV Challenge 50	64	83
Vaccinates					
H654	neg	neg	neg [*]	1:4	1:4
H664	neg	neg	NA	NA	NA
H671	neg	neg	neg	1:8	1:16
H681	neg	neg	neg	1:4	1:8
H684	neg	neg	neg	1:8	1:16
Controls					
H657	neg	neg	NA	NA	NA
H658	neg	neg	neg	1:4	1:16
H677	neg	neg	neg	1:4	1:4
H683	neg	neg	neg	1:4	1:4
H686	neg	neg	NA	NA	NA

* NA, not available due to euthanasia prior to day 50.